Photodynamic Action of Porphyrins on Amino Acids and Proteins. III. Further Studies on the Hematoporphyrin-Sensitized Photooxidation of Lysozyme

Hematoporphyrin-sensitized photooxidation of lysozyme in aqueous solution[1] causes the selective modification of Met-12; the other Met residue, i.e. Met-105, cannot be photooxidized unless the protein molecule has been unfolded prior to irradiation[2,3]. This suggested that, in native lysozyme, Met-12 is more accessible to the photooxidizing agent than Met-105. However, the X-ray structure of lysozyme[4] shows that both Met residues are buried in the internal hydrophobic core.

The possibility remains that the preferential photooxidizability of methionine-12 is due to the intervention of particular mechanistic features in the photoprocess involving this amino acid residue. Therefore, it appeared worthwhile to investigate further the pathway of the aforesaid photosensitized reaction, in order to explore its potential extension to other proteins.

Materials and methods. Irradiations were performed by a 450-watt Xenon lamp[5]. Narrow light bands were isolated by means of Balzers interference filters. In a typical experiment, 8 ml of a 10 μM lysozyme solution in distilled water (pH 5.9) or in 30% acetic acid, added with a 3:1 molar excess of hematoporphyrin, were placed in a Pyrex cuvette of 2 cm light path. The solutions were maintained at 20 ± 1 °C and flushed with purified oxygen. At the end, the sensitizer was removed by gel filtration of the irradiated mixture on Sephadex G-25, using 0.1 M acetic acid as the eluent. The procedures for performing the amino acid analyses and the column chromatography of native and irradiated samples of lysozyme as well as for measuring the enzymic activity, have been described elsewhere[1,2,6]. Mixing experiments to determine the stoichiometry of complexing between lysozyme and hematoporphyrin were performed by the continuous variations method, as described by STEVENS and FELSENFELD[7], i.e. mixtures were made up to contain various proportions of the 2 components, the overall molar concentration being constant.

Results and discussion. At first, we investigated whether an interaction of some sort took place between lysozyme and the ground state of hematoporphyrin. Such a process would presumably alter the absorption spectrum of the dye[8]. Actually, addition of lysozyme to a 5 μM aqueous solution of hematoporphyrin caused a bathochromic shift of the absorption maximum of the Soret band and a noticeable hypochromic effect (Figure 1). The absorbance of the solutions was independent of time after mixing, so that the interaction appeared to be rapid.

In order to get some information about the stoichiometry of the dye-protein complex, the continuous variations method was used, by reading the optical densities of the solutions at 440 nm, where only the complex significantly contributes to absorption. The experimental plot (Figure 2) displayed a single discontinuity at about 65% hematoporphyrin, demonstrating the existence of a 2:1 complex between hematoporphyrin and lysozyme.

On the other hand, no change in the absorption spectrum of aqueous solutions of hematoporphyrin were detected on the addition of various amounts of the single amino acids which are commonly present in protein molecules. Analogously, no interactions between lysozyme and hematoporphyrin appeared to occur in 30% acetic acid solution, i.e. in a medium where the lysozyme molecule is largely disorganized[3]. Therefore, the hematoporphyrin-lysozyme interaction is typical of the native protein, such as to allow the binding of the dye with

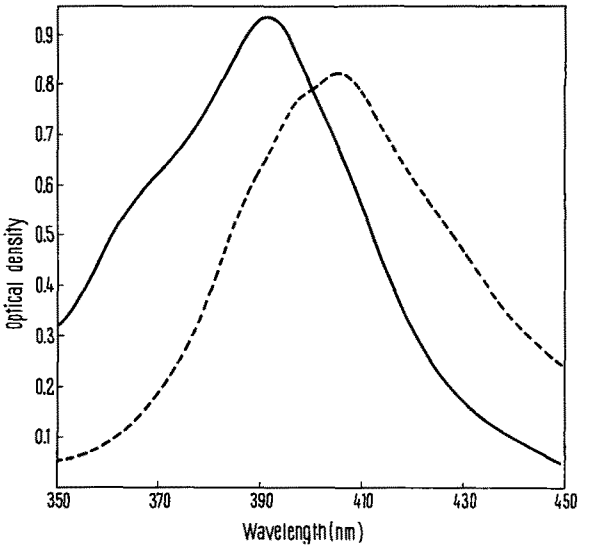

Fig. 1. Absorption spectrum in the Soret region of 5 μM hematoporphyrin (——) and of 5 μM hematoporphyrin plus 5 μM lysozyme (– – –) in aqueous solution, pH 5.9.

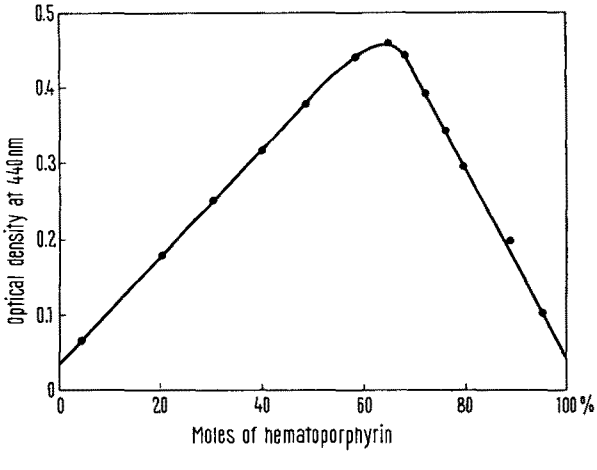

Fig. 2. Mixing curve for hematoporphyrin and lysozyme. The experiments were carried out at room temperature in distilled water, pH 5.9. The closed symbols represent the absorbance at 440 nm.

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determined areas of the lysozyme three-dimensional network.

In order to elucidate the role performed by the aforesaid ground state complex, we carried out some irradiation experiments at 367 nm, where only uncomplexed hematoporphyrin is involved in the light absorption process, and at 435 nm, where the incident light is predominantly absorbed by the complex. The amino acid analyses of lysozyme after 30 min irradiation in aqueous solution are reported in the Table. Apparently, the 367 nm light brought about no change in the amino acid composition of the protein; moreover, the isolated product possessed enzymic activity, UV-absorption spectrum, and chromatographic behaviour on Amberlite CG-50 coincident with those of unirradiated lysozyme. On the other hand, when the 435 nm light was used, one Met residue was converted to the sulfoxide: the enzymic activity (52%), as well as the spectral and chromatographic parameters, measured for this sample were identical with those previously determined for Met-12 sulfoxide-lysozyme, prepared by irradiation of the hematoporphyrin-lysozyme system with white light[1-3].

Now, when free Met or the dipeptide Z-Met-Asp were exposed to 367 nm light in the presence of hematoporphyrin, a fast and quantitative conversion of the thioether function to the sulfoxide was achieved. Furthermore, on illumination of lysozyme plus hematoporphyrin at 367 nm in 30% acetic acid solution, both the Met residues were photooxidized. It is apparent that the 367 nm light can promote the transition of free hematoporphyrin to the excited state which is responsible for photoactivating molecular oxygen to attack the methionine sulfur. Therefore, the lack of photooxidation of Met-12 by free hematoporphyrin, when lysozyme retains its native conformation, is to be ascribed to the burial of this residue inside the protein molecule which prevents direct interaction between the Met side chain and the dye dissolved in the aqueous solvent. On the other hand, the fact that only light absorbed by complexed hematoporphyrin is effective in inducing the photooxidation of Met-12 can be easily interpreted if the region involving this amino acid is assumed to be one of the two binding sites of the dye. In this case, the protein conformation exerts no shielding to the contact between the Met side chain and the photooxidizing agent; moreover, the high local concentration of sensitizer should greatly enhance the efficiency of the photooxidative reaction, as it has been actually observed. The second molecule of hematoporphyrin bound per molecule of lysozyme appears to interact with a site (or, possibly, to be distributed among sites) not containing any of the amino acids photooxidizable under our conditions.

These findings point out that caution must be exercised in drawing conclusions about the degree of exposure of amino acid residues in proteins from photooxidative investigation, before the detailed mechanism of the photoreaction has been cleared up. Our results open interesting prospects in the field of the dye-sensitized photooxidation of proteins, since by a procedure similar to that employed in the present study, the photodynamic action of dyes can be restricted within selected areas of protein molecules[9]; this should allow one selectively to modify a limited number of amino acid residues and to obtain some information about the nature of the binding sites of dyes[10].

Amino acid analyses of 30 min irradiated lysozyme

Amino acid	Irradiation wavelength		
	367 nm (water, pH 5.9)	435 nm (water, pH 5.9)	367 nm (30% acetic acid)
Tryptophan	6.0	5.9	5.9
Histidine	0.8	0.9	0.8
Tyrosine	3.1	2.9	3.0
Methionine	2.1	1.0	0.0
Methionine sulfoxide	0.0	0.9	2.0

All the amino acids which are present in lysozyme were examined, but the table reports only those which are known to be susceptible of photooxidation[1]. No change was found in the content of the other amino acid residues.

Riassunto. L'ematoporfirina forma con il lisozima un complesso in rapporto molare 2:1. Uno dei siti di legame è rappresentato dalla regione della molecola proteica contenente la metionina-12. L'irradiamento con luce assorbita solo dall'ematoporfirina complessata consente, quindi, la fotoossidazione selettiva di tale amminoacido.

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On the Origin of D-Aminoacid Residues in Microbial Peptides

Peptides isolated from micro-organisms frequently contain residues of D-aminoacids. Several studies have shown that these residues are derived biogenetically from free L-aminoacids, the free D-enantiomers being generally poor precursors[1]. Accordingly, inversion must occur after the L-aminoacid has been converted to a derivative. Two possibilities may be envisaged. Firstly, derivatization of the carboxyl group to form an activated compound may be accompanied or followed by racemization or inversion, the resulting activated D-aminoacid then being used to synthesize the peptide. Alternatively, the L-aminoacid may be incorporated as an L-residue which later undergoes inversion. We here present evidence for operation of the latter mechanism during biosynthesis of analogues of the cyclotetradepsipeptide, angolide, by the fungus *Pithomyces sacchari* (Speg.) M. B. Ellis, IMI 120725.

The organism was grown in surface culture on a glucose-salts-peptone-agar medium containing L-valine (12.5 mg/ml). After incubation for 2 weeks at 25 °C the angolide fraction[2] was isolated, and its properties were compared with those of authentic angolide[3]. The results